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Comparative analysis of *Fusarium graminearum* infection in maize, wheat, and rice

Análisis comparativo de la infección por *Fusarium graminearum* en maíz, trigo y arroz

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Abstract

Plants such as maize, wheat, and rice, belonging to the grass family, are of great economic importance as they are the basis of world nutrition. Therefore, it is out most important to study and report pathogens that put this group of plants at risk, because they cause diseases that result in decreased product quality and loss of commercial yield. *Fusarium graminearum* is a necrotrophic fungus that causes Fusarium head Blight in wheat and Cob rot in maize. In the present research we determined and compared the pathogenicity of hyper and hypo-virulent mutants with the wild-type strain of *F. graminearum* on maize, wheat, and rice. For this purpose, we employed techniques such as genomic DNA isolation, polymerase chain reaction (PCR), seed germination under greenhouse and laboratory conditions, inoculation of conidia in suspension in seedlings and seeds as well as the use of epifluorescence microscopy. Our results demonstrated first, the strains used in this study have the expected genotype; second, *F. graminearum* does not present an effect on seed germination; and third, *F. graminearum* can penetrate and grow in maize and wheat, but in rice results were inconclusive.

Key words: *Fusarium graminearum*; infection, maize, wheat, rice.

Introduction

The grass family (Poaceae) consists of monocotyledonous plants with great importance because crops such as maize, wheat, and rice, belonging to this family, are the basis of food worldwide. These are used either directly, in the form of cereal grains, flours, and oils, or indirectly as livestock feed (Sánchez, 2019). Maize (*Zea mays*) plants, in addition providing nutrients to animals and humans, is a basic raw material for industry. According to its production, maize is divided into yellow and white, where yellow is mainly used for the manufacture of balanced food and livestock feed, while white maize is mostly for human consumption (ASERCA, 2018). The annual production of maize grain is 850 million tons, and among the 10 main producing countries are the United States, China, Brazil, Argentina and Mexico in America, and China, India, and Indonesia in Asia (McCormick, 2020). Wheat (*Triticum aestivum*) is considered a strategic product because it is one of the most important resources in the diet due to its high nutritional content. It is a crop that develops best in heavy soils, with abundant marl (type of sedimentary rock) and clay, in regularly temperate and cold subtropical climates (SAGARPA, 2016). World wheat production in 2022-2023 is estimated to increase 1.26 million tons (0.16%). Countries like China (138 million metric tons), the European Union (134.3 million metric tons) and India (103 million metric tons) top the list of largest wheat producers in the world. Mexico is in the 21st place worldwide, producing 3.5 million metric tons (World Agricultural Production, 2022). Rice (*Oryza sativa*) is a cereal that occupies first place as a staple food, if it is considered that it provides more calories per hectare than any other crop, however, considering the harvested area, it occupies second place. In the Mediterranean region of southern Europe, rice cultivation is intensive, while in Africa and America it is also widely cultivated. It is the typical cereal of South and East Asia, providing employment to the largest sector of the rural population. Rice cultivation is considered from tropical to subtropical, so its growth temperature ranges from 10 °C to 40 °C, considering optimum temperatures of 30-35 °C (Ampong-Nyarko and De Datta, 1991).

F. graminearum is a necrotrophic fungus that mainly affects the grass family and specifically infects seedlings, grains, ears, roots, and stems (Takemura *et al*., 2007). *F. graminearum* is a terrible threat to the world economy since it causes severe symptoms such as shrinkage of the seeds contaminating them with mycotoxins, decreasing the quality of the product and causing a reduction in commercial yield (Montiel-González *et al*.,

2005; Hernández *et al*., 2007; Lamprecht *et al.,* 2011). In a study that discusses the impact of the enzymes deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DOHH) on the life cycle of *F. graminearum*, it was concluded that DHS overexpression leads to increased number of infection structures, faster invasion of wheat tissue and increased virulence in wheat. In contrast, DOHH overexpression completely prevents the formation of infection structures and pathogenicity in wheat (Martinez-Rocha *et al*., 2016). In this study we analyzed the effect of infection of the wild-type strain (WT) and a hyper virulent mutant (DHSoex), and a hypo virulent mutant (DOHHoex) of *F. graminearum* in the most representative crops of the world economy and food, maize, wheat, and rice.

Methods

Fungal strains and culture conditions. The strains used in this study were wild-type strain (WT: Fg 8/1; Miedaner *et al*., 2000) and overexpressing mutants DHSoex and DOHHoex from *F. graminearum* (Martínez-Rocha *et al.,* 2016) containing the GFP gene expressed constitutively. The strains grew on Potato Dextrose Agar (PDA) plates, at 28°C for 2 to 3 days. To produce mycelia, 1 cm^3 was transferred into a flask with 20 mL of PDB medium and cultured at 28 °C, with 180 rpm shaking for 2 days. To produce conidia 1 cm^3 of fungal mycelia was transferred into a flask with 20 mL wheat medium with 20 µL ampicillin (100 mg/mL) and cultured at 28°C for 5 days. Conidia of *F. graminearum* strains were collected by filtration and centrifugation at 3000 *x g* for 15 min at 10°C washed with 20 mL of sterile distilled water, resuspended in 1 mL of sterile water, and finally counted using a Newbauer hemocytometer, and the concentration adjusted to 1 x 10^6 conidia/mL.
Plants growth conditions. Maize (*Zea mays*), wheat (*Triticum aestivum*), or rice (*Oryza sativa*) seeds were sterilized using 0.5% sodium hypochlorite (Cloralex) and washed with sterile distilled water. Germination and growth were carried out in seedbeds with 50 pots. Two maize seeds or four wheat or rice seeds were placed in each pot containing vermiculite. All of them were irrigated with sterile distilled water and put to germinate in the greenhouse at a temperature between 18 °C to 38 °C. Germination time was 7 days for maize and wheat and 13 days for rice.
DNA extraction and PCR conditions. Genomic DNA extraction was prepared according to Aljanabi *et al*., (1997). Briefly, mycelia of *F. graminearum* strains were grinded with liquid nitrogen and placed in 2 mL tubes adding 0.9 mL extraction buffer (0.4M NaCl, 10mM Tris-HCl pH=8, 2mM EDTA), 8 µL protease K and incubated at 60° C for 1 h. 320 µL of 5M NaCl solution were added, then vortexed for 30 seconds and centrifuged for 30 min at 10000 rpm. The supernatant was collected and poured into a new tube adding one volume of isopropanol, incubating for 10 minutes at RT, and centrifuged for 20 min at 10000 rpm. Subsequently, the pellet was washed with 500 µL of 80% EtOH and dried for 10 min. Pellet was resuspended in 100 µL of milliQ H_2O and treated with 4 µL RNAse. 2 µL were quantified by NanoDrop. Amplification of DNA fragments of DHS or DOHH genes were prepared by PCR using the extracted genomic DNA samples as templates with specific primers (Table 1), and the DreamTaq Polymerase (PROMEGA) using the manufacturers conditions. PCR conditions were initial denaturation of 95 °C/3 min, 35 cycles of 95 °C/30 s, 62 °C/20 s, 72 °C/ 30 s, and termination of 72°C/ 5 min and keep at 16°C. Finally, the samples were visualized by electrophoresis using a 1.5% agarose gel.
***F. graminearum* infection seeds.** 10 maize or 15 wheat or rice seeds were placed in a Petri dish with two Whatman papers soaked in 5 mL sterile distilled water. 200 µL of a conidial suspension of 5 $X10^3$ or $2X10^4$ conidia/mL were placed on each seed. Plates with inoculated seeds were incubated at 28 °C for 2 days.
Microscopy of infected plants. Sheath leaves of maize, wheat, and rice were prepared and inoculated with 5 µL of 5 $X10^3$ conidia/mL conidial suspension of WT-GFP strain, DHSoex-GFP, or DOHHoex-GFP overexpressing mutants. The inoculated sheath leaves were placed on a petri dish with sterile water to secure a high relative humidity for conidia germination. After 2 to 3 days post-inoculation (dpi), the samples were cut longitudinally with a razor blade, and histological preparations were observed under the epifluorescence microscope (Zeiss-Axioskop 40). The GFP was excited with a wavelength of 450 nm and detected at 500 to 550 nm. Images were taken with an AxioCam MRc (Zeiss) camera. Images processing was done with Zeiss AxioVision SE64 software (version 4.8.1).

Results and Discussion

To determine de genotype of the WT strain and DHSoex and DOHHoex mutants, fragments of the DHS and DOHH genes under the strong and constitutive promoters PgpdA and Pgpd1, respectively, were amplified using PCR. Primers used in this study are shown in Table 1.

Table 1. *Primers used for PCR in this study.*

Gene	Primer	Length	Tm °C	% GC	Sequence (5´ → 3´)	Amplified product from gDNA (pb)
DHS	1F-PgdpA	20	62°C	55%	AAGGTCGTTGCGTCAGTCCA	555
	1F-DHS	20	62°C	55%	ATGGCCTCCAACTCTGATGC	293
	2qR-DHS	20	62°C	55%	GACCCGACGAGATGAGATTG	
DOHH	1F-Pgdp1	20	62°C	55%	CATGTTGCGAGGTGGGTGAT	722
	1F-DOHH	21	62°C	47.6%	ATGTCGCCTTCTGCTGATACT	312
	2R-DOHH	20	62°C	55%	GGGGTCCTCCTTAAGATCAG	

Results demonstrated that DHSoex mutant contains the DHS gene under PgpdA promoter due to a fragment of 555 pb using 1F-PgpdA and 2R-DHS was amplified when DHSoex, but not when WT gDNA was used. PCRs with internal DHS primers (1F-DHS + 2R-DHS) were used as a positive control for amplification (293 pb) with both WT and DHSoex gDNA and distilled sterile water was used as negative control template (Figure 1D). Similarly, results demonstrated the DOHHoex mutant genotype because a fragment of 722 pb was amplified using gDNA from DOHHoex but not with WT gDNA. Positive controls amplified a fragment of 312 pb using internal primers of the DOHH gene and WT or DHSoex gDNA. Distilled sterile water was used as a negative control (Figure 1E). It is important to note that a double band was amplified when the DHSoex gDNA and internal primers were used, this could be due to a DHS gene duplication. However, this duplication does not affect the DHSoex mutant overexpressing phenotype.

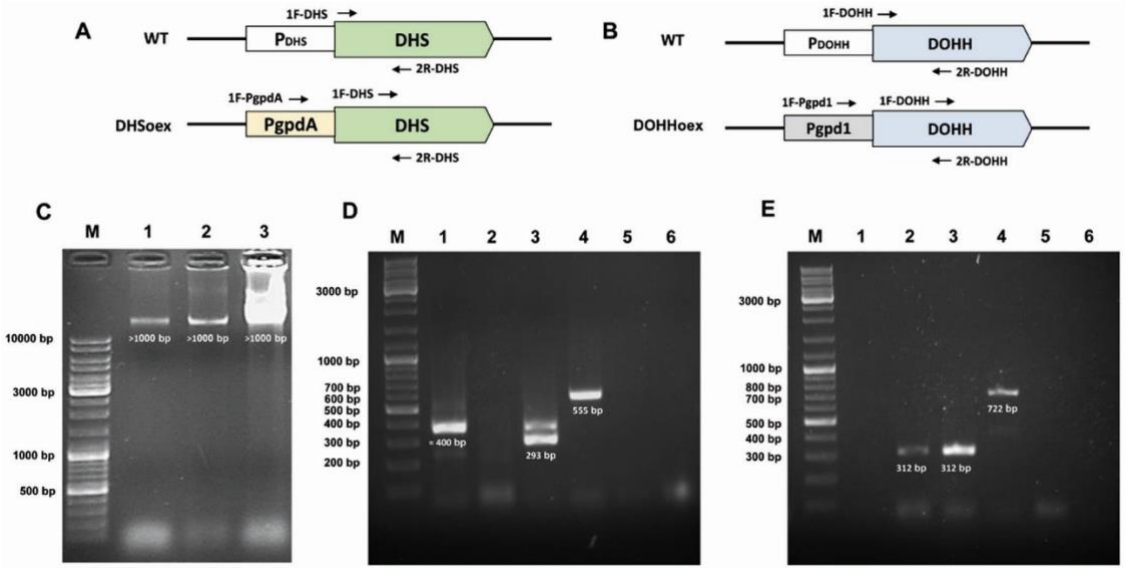

Figure 1. Genotype verification of strains used in this study. *(**A**) Schematic representation of DHS allele in WT (upper image) and DHS overexpressing mutant allele (lower image). (**B**) Schematic representation of DOHH allele in WT (upper image) and DOHH overexpressing mutant allele (lower image). (**C**) Visualization of genomic DNA by electrophoresis on an agarose gel 1%; 1: WT strain, 2: DHS mutant, 3: DOHH mutant. (**D**) Visualization of DHS gene fragments amplified by PCR using: 1: WT gDNA with 1F-DHS + 2R-DHS, 2: WT gDNA with 1F-PgdpA + 2R-DHS, 3: DHSoex gDNA with 1F-DHS + 2R-DHS, 4: DHSoex gDNA with 1F-PgdpA + 2R-DHS, 5: H_2O (Negative control) with 1F-PgdpA + 2R-DHS and 6: H_2O (Negative control) with 1F-DHS + 2R-DHS. (**E**) Visualization of DOHH gene fragments amplified by PCR using: 1: WT gDNA with 1F-Pgdp1 + 2R-DOHH primers, 2: WT gDNA with 1F-DOHH + 2R-DOHH, 3: DOHHoex gDNA with 1F-DOHH + 2R-DOHH, 4: DOHHoex gDNA with 1F-Pgdp1 + 2R-DOHH, 5: H_2O (Negative control) with 1F-DOHH + 2R-DOHH, 6: H_2O (Negative control) with 1F-Pgdp1 + 2R-DOHH.*

To determine the effect of *F. graminearum* WT strains and DHSoex or DOHHoex mutants' infection on maize, wheat, and rice seeds we sterilized the seeds with a 5% solution of NaClO for 15 minutes and washed twice to avoid contamination. Seeds were distributed in 90 mm Petri dishes containing filter paper soaked with 5 ml

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of sterile distilled water. We used 200 μL of conidial suspensions ($5x10^3$ or $2x10^4$ conidia/mL) of *F. graminearum* WT, DHSoex, and DOHHoex strains and water as a negative control to infect the surface of the seeds. Infected seeds were incubated at 28°C for 48 hours. Table 2 summarizes the germination results, where the highest percentage of germination is in wheat seeds (93 %), followed by maize seeds (50 %) and rice seeds (47 %).

Table 2. *Germination rate of maize, wheat and rice seeds exposed to F. graminearum WT strain, DHSoex or DOHHoex mutants.*

Plant	Dilution (Conidia/mL)	Strain	Total seeds (n=)	Sprouted seeds (n=)	Germination rate (%)
Maize	$5x10^3$	H_2O	10	1	10
		WT	10	4	40
		DHSoex	10	4	40
		DOHHoex	10	2	20
	$2X10^4$	H_2O	10	5	50
		WT	10	5	50
		DHSoex	10	3	30
		DOHHoex	10	1	10
Wheat	$5x10^3$	H_2O	15	14	93
		WT	15	13	87
		DHSoex	15	13	87
		DOHHoex	15	13	87
	$2X10^4$	H_2O	15	14	93
		WT	15	12	80
		DHSoex	15	12	80
		DOHHoex	15	14	93
Rice	$5x10^3$	H_2O	15	6	40
		WT	15	7	47
		DHSoex	15	12	80
		DOHHoex	15	7	47
	$2X10^4$	H_2O	15	7	47
		WT	15	5	33
		DHSoex	15	9	60
		DOHHoex	15	5	33

At the time of observation of the seeds, a higher level of infection was observed in the maize seeds with a white and slightly brown coleoptile coloration; on the other hand, the wheat seeds showed no signs of infection (Figure 2). At the end of the experiment, it is not entirely clear if there is a correlation between the infection of the *F. graminearum* strains and the germination of the seeds since the total germination in the negative control was not conclusive to have a margin of comparison between this and those exposed to the strains of *F. graminearum*.

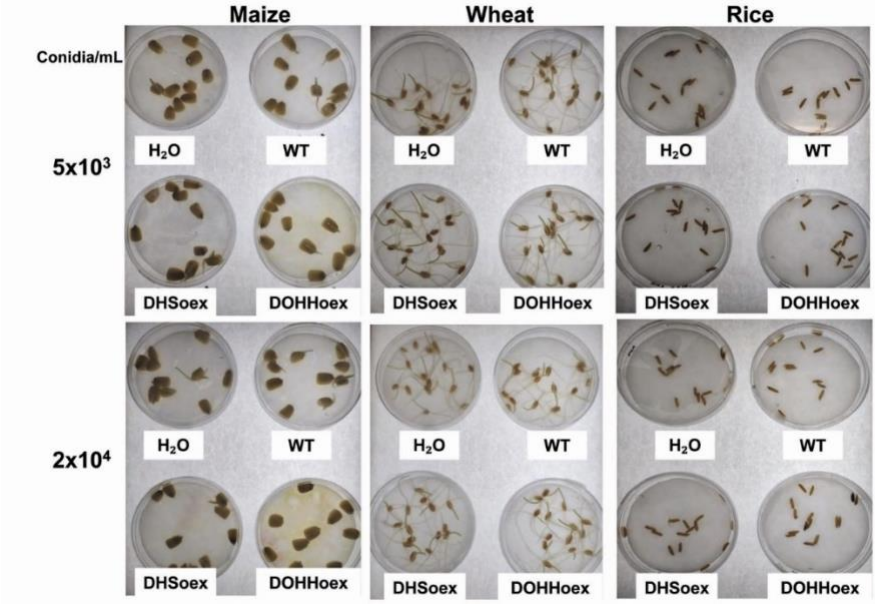

Figure 2. Effect of F. graminearum infection on germination of maize, wheat, and rice seeds. *Maize, wheat, and rice seeds were inoculated with 200 microliters of each strain dilution (2x104 and 5x103 conidia per ml). The strains used in dilutions were WT, DHSoex, DOHHoex. Water was used as a negative control. They were incubated for 48 hours at 28°C. Photographs were taken 2 days after inoculation. Infection was detected mainly in*

maize seeds.

Sheath leaves of maize, wheat, and rice were inoculated with 5 µL of conidial suspension (5 X10^3 conidia/mL) of WT-GFP strain, DHSoex-GFP, or DOHHoex-GFP mutants, along with sterile distilled water as a negative control. In the negative control, only plant cell wall (green) and chloroplasts (red) autofluorescence was observed under the GFP channel. Microscopic observation showed that hyphal density in maize sheath leaves inoculated with WT strain was scarce, but the hyphae have penetrated the maize cells. The DHSoex mutant additionally to penetration presents higher germination than the WT strain. In contrast, the DOHHoex mutant germinates profusely but hyphae were vacuolated and did not penetrate the plant cells (Figure 3A). Similar results were identified in wheat sheath leaves, the WT strain infection was hardly seen, however the observed hypha has already penetrated the plant cells. In the DHSoex mutant hyphae development was higher compared to the WT strain. Meanwhile, the DOHHoex mutant only had a slight growth of vacuolated epiphytic hyphae (Figure 3B). On the other hand, *F. graminearum* WT strain and DHSoex, DOHHoex mutants showed no infection in rice sheath leaves. The microscopic images revealed that the plant cells were not affected after inoculation, and there was no sight of hyphae above or beneath the samples (Figure 3C). These results suggest that *F. graminearum* DHS overexpressed mutant is significantly more virulent in maize and wheat. While the DOHH overexpressed mutant showed weak superficial colonization and lack of proliferation in both maize and wheat. Finally, none of them succeeded in infecting rice sheath leaves.

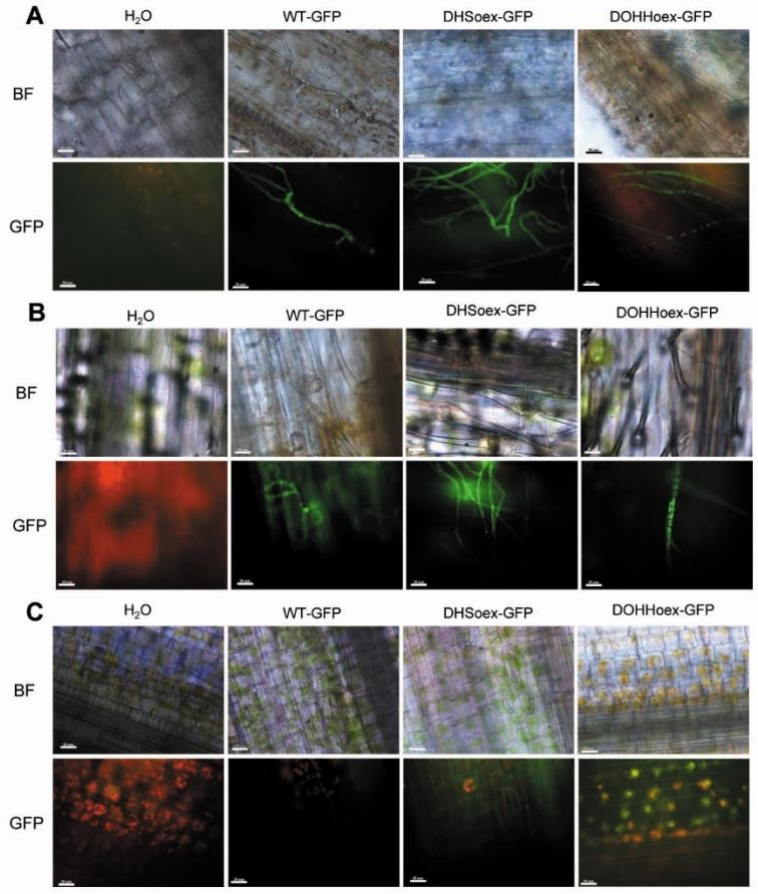

Figure 3. Fluorescently tagged F. graminearum strain infection in maize, wheat, and rice sheath leaves. *Microscopic images of maize, wheat, and rice sheath leaves were taken 3 days post-inoculation with WT-GFP strain, DHSoex-GFP and DOHHoex-GFP mutants of F. graminearum. (**A**) The WT-GFP strain and DHSoex-GFP mutant show great hyphal invasion in maize, whereas the DOHHoex-GFP mutant hyphae were vacuolated. (**B**) The microscopic images of wheat samples revealed WT-GFP strain inner infection as well as DHSoex-GFP mutant, but in higher degree. The DOHH mutant was barely seen. (**C**) There was no sight of infection in rice sheath leaves. Distilled sterile water was used as a negative control. Images were taken with a Zeiss-Axioskop 40 microscope in Bright Field and GFP Chanel. White bar = 20 µm*

Conclusions

The results of the PCR amplification demonstrated that the genotype of the strains used in this study was as expected, since the DHS and DOHH genes are located under the strong and constitutive PgpdA and Pgpd1 promoters, respectively. The effect of the wild-type strain and overexpressed mutants DHSoex and DOHHoex of *F. graminearum* infection on the germination of maize, wheat and rice seeds did not present conclusive results due to germination rate of the seeds without fungus was very low. Through microscopic observation of the different plants inoculated with *F. graminearum*, we verified that maize and wheat plants are more susceptible to infection. In contrast, in rice plants mycelium did not appear inside.

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